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Identification of a Plasmid-Borne Parathion Hydrolase Gene from *Flavobacterium* sp. by Southern Hybridization with *opd* from *Pseudomonas diminuta*†

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Parathion hydrolases have been previously described for an American isolate of *Pseudomonas diminuta* and a Philippine isolate of *Flavobacterium* sp. (ATCC 27551). The gene which encodes the broad-spectrum organophosphate phosphotriesterase in *P. diminuta* has been shown by other investigators to be located on a 66-kilobase (kb) plasmid. The intact gene (*opd*, organophosphate-degrading gene) from this degradative plasmid was cloned into M13mp10 and found to express parathion hydrolase under control of the *lac* promoter in *Escherichia coli*. In *Flavobacterium* sp. strain ATCC 27551, a 43-kb plasmid was associated with the production of parathion hydrolase by curing experiments. The M13mp10-cloned fragment of the *opd* gene from *P. diminuta* was used to identify a homologous genetic region from *Flavobacterium* sp. strain ATCC 27551. Southern hybridization experiments demonstrated that a genetic region from the 43-kb *Flavobacterium* sp. plasmid possessed significant homology to the *opd* sequence. Similar hybridization did not occur with three other native *Flavobacterium* sp. plasmids (approximately 23, 27, and 51 kb) present within this strain or with genomic DNA from cured strains. Restriction mapping of various recombinant DNA molecules containing subcloned fragments of both *opd* plasmids revealed that the restriction maps of the two *opd* regions were similar, if not identical, for all restriction endonucleases tested thus far. In contrast, the restriction maps of the cloned plasmid sequences outside the *opd* regions were not similar. Thus, it appears that the two discrete bacterial plasmids from parathion-hydrolyzing soil bacteria possess a common but limited region of sequence homology within potentially nonhomologous plasmid structures.

Organophosphate insecticides such as parathion (*O,O*-diethyl-*O*-nitrophenyl phosphorothioate) and related phosphorothioates have been used increasingly over the last decade as replacements for the more persistent chlorinated hydrocarbon pesticides. The relative lack of persistence of some organophosphates has been attributed to their susceptibility to hydrolysis by microbial enzymes. Recent reports of the reduced efficacy of these compounds in so-called problem soils, where pesticide metabolism is apparently enhanced, may be due to the increasing prevalence of these hydrolytic enzymes (22). The nature of this enhanced metabolism, as well as the potential use of microbial degradative enzymes for pesticide waste detoxification (20), has led to heightened interest in characterizing the genetic and enzymatic components of these hydrolases.

The microbial degradation of parathion has received considerable attention because of both its widespread use and the ready detection of its hydrolytic products (*p*-nitrophenol and diethylthiophosphoric acid) (21). Parathion hydrolase activity has been investigated with pure cultures of an American isolate of *Pseudomonas diminuta* (26) and a Philippine *Flavobacterium* sp. (ATCC 27551) (6, 27, 28). In both cases, the hydrolases were constitutively expressed and displayed similar substrate specificity toward structurally related organophosphates. In neither case were the bacteria able to use parathion as the sole carbon source.

In *P. diminuta*, parathion hydrolase (EC 3.1.3) is encoded on a plasmid of approximately 66 kilobases (kb) (26). When the degradative bacterial host was cured of this plasmid by growth in the presence of mitomycin C, parathion hydrolase activity was lost (26). The plasmid-borne gene (defined here as *opd* for *o*rgano*p*hosphate *d*egradation, in consultation with Bruce Holloway, Monash, Australia) encodes the broad-spectrum organophosphate hydrolase and has been cloned into other bacterial hosts on various plasmids (25) and several phages (C. S. McDaniel, J. R. Wild, and G. A. O'Donovan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H159, p. 134). These heterologous genetic constructions were mapped by restriction endonuclease analysis, and the tentative gene organization was determined.

In *Flavobacterium* sp. strain ATCC 27551, the genetic organization and location of the hydrolase gene is unknown. Furthermore, the relationship of the *opd* genes from these two bacteria has not been evaluated. Brown (6) has estimated the molecular weight of the *Flavobacterium* sp. enzyme from a crude preparation to be 50,000 daltons. Recently, it has been estimated that the product of *opd* in *P. diminuta* is a 30,000-dalton protein (C. S. McDaniel, Ph.D. thesis, Texas A&M University, College Station, 1985). The object of the current research is to define the genetic location of the *opd* gene from the *Flavobacterium* sp. as a first step toward comparing the genes encoding the enzymes which hydrolyze this important class of organophosphate pesticides and initiate an investigation of their possible involvement in problem soils. Specifically, we sought to (i) determine whether the hydrolase is plasmid-encoded in

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Flavobacterium sp. strain ATCC 27551 and (ii) determine whether the hydrolase gene in this *Flavobacterium* sp. is homologous to the *opd* gene in *P. diminuta*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Flavobacterium* sp. strain ATCC 27551 and plasmid-cured derivatives were grown in nutrient broth (Difco Laboratories, Detroit, Mich.) at 30°C. *Escherichia coli* HB101 (5) and JM103 (19) were grown in L broth (16) at 37°C. *P. diminuta* PD3 (obtained from D. Gibson and C. Serdar) was grown in L broth at 32°C. *Flavobacterium* sp. strains S101 through S105 were derived from *Flavobacterium* sp. strain ATCC 27551 after streptomycin sulfate (Sm) treatment as described below. pBR325 (3) was kindly provided by M. Voll. pBR322 (4) and M13mp10 (19) were obtained from Bethesda Research Laboratories, Bethesda, Md.

DNA isolation. *Flavobacterium* sp. total cellular DNA was isolated by the method of Marmur (18). *Flavobacterium* sp. plasmid DNA was isolated by the method of Casse et al. (8). A mild lysis procedure (2) was used to obtain plasmid DNA from *P. diminuta* PD3. Plasmid preparations from both strains were purified by cesium chloride isopycnic centrifugation. Plasmid DNA was isolated from *E. coli* HB101 by the rapid boiling technique (15). M13 replicative form (RF) and single-stranded DNAs were isolated from infected *E. coli* JM103 cells by the methods in the M13 cloning manual (Bethesda Research Laboratories).

Isolation of streptomycin-resistant mutants. Nutrient agar plates containing 500 μg of streptomycin per ml were inoculated with *Flavobacterium* sp. strain ATCC 27551 which had been grown for 48 h in nutrient broth. After 4 days, streptomycin-resistant (Sm^r) colonies were picked away from a light background lawn of Sm^s cells with a sterile toothpick and used to inoculate another streptomycin plate. From this plate cells were taken to inoculate nutrient broth cultures for use in further studies.

Cloning of the *P. diminuta opd* gene. Plasmid pCMS1 (C. M. Serdar [26]) was isolated from *P. diminuta* PD3 and subjected to digestion with *Pst*I as described by the manufacturer (Bethesda Research Laboratories). The *Pst*I fragments were ligated to *Pst*I-digested pBR322, inactivating the ampicillin resistance gene. The resulting plasmid mixture was transformed into $CaCl_2$-competent strain HB101 cells (16) and tetracycline-resistant, ampicillin-sensitive colonies were selected by replica plating. A single colony exhibiting parathion hydrolase activity was isolated, and plasmid DNA was purified by cesium chloride density centrifugation. The plasmid insert was subcloned into M13mp10 by purifying *Pst*I restriction fragments from recombinant plasmids (24) and ligating them between the operator-promoter of *lac* and its proximal *lacZ* (19).

Cloning of the *Flavobacterium* sp. *opd* gene. Purified *Flavobacterium* sp. plasmid DNA was subjected to partial digestion with *Eco*RI (200 μg of DNA per ml, 0.8 U of enzyme per μg of DNA, 5 min at 18°C), ligated to *Eco*RI-digested pBR325 DNA (40 μg/ml DNA concentration, 1:1 ratio of vector to insert DNA, 14°C, 18 h, 200 U of DNA ligase [Boehringer-Mannheim] per ml), and used to transform competent *E. coli* HB101 cells. Ampicillin-resistant, chloramphenicol-sensitive colonies were isolated and assayed for parathion hydrolase activity (25). Broth cultures of these recombinant strains were grown for small-scale plasmid isolation.

DNA-DNA hybridization. Undigested and digested total cellular and plasmid DNAs from *Flavobacterium* were separated by electrophoresis on horizontal 0.7% agarose gels with 40 mM Tris–20 mM acetic acid–2 mM EDTA (pH 7.8)–0.5 μg of ethidium bromide per ml as gel and electrode buffers. Lambda phage DNA digested with *Hin*dIII was used as a standard for molecular weight determinations of linear DNA fragments. DNA transfer and filter hybridization were performed by a modification of the method of Southern (29) with GeneScreen hybridization transfer membranes (New England Nuclear Corp.). Stringent conditions were achieved by performing the hybridizations and subsequent washes at 65°C with dextran sulfate by the manufacturer's directions for GeneScreen. To prepare the *opd* probe, M13-008 RF was digested with *Pst*I, and the 1.3-kb fragment containing the *opd* gene from *P. diminuta* was isolated by electrophoresis followed by electroelution from excised gel fragments into a dialysis bag (16). The 1.3-kb fragment was labeled with [^{32}P]dCTP with a nick-translation kit (Bethesda Research Laboratories) (17).

RESULTS

Curing of *Flavobacterium* sp. plasmids and *opd* activity. *Flavobacterium* sp. strain ATCC 27551 contains four plasmids with approximate sizes of 23, 27, 43, and 51 kb. Treatment of this strain with the plasmid-curing agents mitomycin C, novobiocin, ethidium bromide, and sodium dodecyl sulfate (7) did not result in any appreciable number (<1%) of cells lacking parathion hydrolase activity (data not shown). However, selection of spontaneous Sm^r mutants resulted in a large proportion of cells that had lost one or more of their plasmids. Approximately 50% of Sm^r mutants irreversibly lost the ability to hydrolyze parathion. Figure 1 shows the plasmid profiles from five Sm^r mutants, two of which possessed hydrolase activity and three of which had lost it. Analysis of the plasmid profiles from 52 Sm^r mutants revealed that loss of hydrolase activity was coincident with the loss of the 43-kb plasmid.

Cloning of the *opd* gene sequence from *P. diminuta*. A 1.3-kb *Pst*I restriction fragment from pCMS1 was cloned

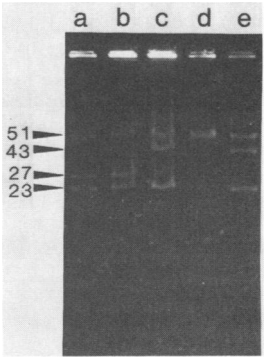

FIG. 1. Plasmid content of parathion hydrolase-positive and -negative derivatives of *Flavobacterium* sp. strain ATCC 27551 isolated after streptomycin treatment. Plasmid DNA was isolated and subjected to electrophoresis as described in the text. Values for plasmid molecular size, expressed in kilobases, were determined by comparing the migration of *Flavobacterium* sp. plasmid DNA relative to that of plasmids of known molecular weight (RP-4 [23], R68.45 [14], and pLAFR1 [12]). Lanes: a, S101 (hydrolase negative); b, S102 (hydrolase negative); c, S103 (hydrolase positive); d, S104 (hydrolase negative); and e, S105 (hydrolase positive).

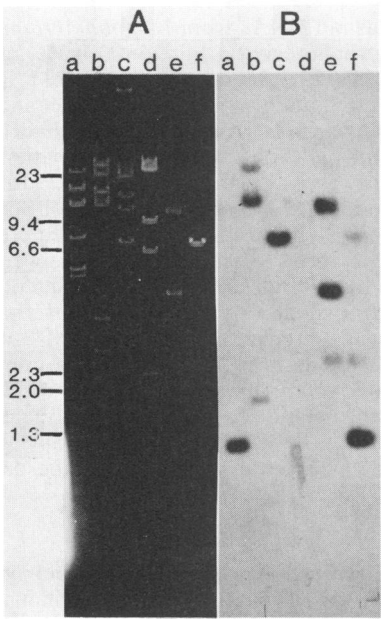

FIG. 2. Hybridization of a ^{32}P-labeled *Pst*I fragment encoding the *opd* gene from *P. diminuta* to digested plasmid DNA from *Flavobacterium* sp. strain ATCC 27551. (A) Agarose gel electrophoresis of digested plasmid DNA. (B) Corresponding autoradiogram after transfer of the DNA fragments to a membrane and hybridization to the *opd* probe. Plasmid DNA was digested with *Pst*I (lane a), *Sal*I (lane b), and *Eco*RI (lane c). Lane d, *Hin*dIII-digested lambda phage DNA; lane e, undigested M13-008 RF DNA; lane f, *Pst*I-digested M13-008 RF DNA.

into pBR322 and expressed in *E. coli* at low levels relative to its native location in *P. diminuta* (data not shown). The productive *opd* sequence was removed from this recombinant plasmid (MCD038) by *Pst*I digestion, and the purified fragment was introduced into the *Pst*I site of M13mp10. The resulting recombinant DNA molecules were transformed into competent JM103 cells, and selected single plaque isolates were grown in 5-ml roller tube cultures. Approximately 50% of these recombinant phage molecules expressed *opd* and produced parathion hydrolase. A single plaque isolate (M13-008) containing the 1.3-kb fragment and encoding parathion hydrolase was selected as the representative *opd*-containing phage. The nonexpressing recombinants were demonstrated to have oppositely oriented inserts by complementation testing (data not shown).

DNA-DNA hybridization. With a nick-translated 1.3-kb *Pst*I fragment containing the *opd* gene from *P. diminuta* as a probe, a Southern hybridization experiment was conducted against total cellular and cesium chloride-purified plasmid DNA from *Flavobacterium* sp. Sm^r mutants (Fig. 2). The probe did not hybridize to total cellular DNA from two hydrolase-negative strains, but it did hybridize to total cellular DNA from two hydrolase-positive strains (data not shown). Digestion of purified plasmid DNA containing all four native plasmids from *Flavobacterium* sp. strain ATCC 27551 (hydrolase positive) with restriction endonucleases yielded fragments that showed strong hybridization with the probe. Specific fragments showing hybridization were a 1.3-kb *Pst*I fragment (Fig. 2, lane a), 1.9-, 13.6-, and 31-kb *Sal*I fragments (lane b), and a 7.3-kb *Eco*RI fragment (lane c). Thus, specific restriction fragments of *Flavobacterium* sp. plasmid DNA displayed homology to the *opd* gene from *P. diminuta*. By cloning and restriction mapping the 7.3-kb *Eco*RI *Flavobacterium* sp. plasmid fragment (described below), we were able to account for the hybridization of each of these fragments except the 31-kb *Sal*I fragment. This band was probably the result of incomplete digestion of the plasmid DNA. That the two *opd* genes might share extensive homology was suggested by the observation that both genes were encoded on equal-sized *Pst*I fragments (compare lanes a and f).

Cloning and mapping the *Flavobacterium* sp. *opd* gene. The above curing and hybridization experiments strongly suggested that the *Flavobacterium* sp. *opd* gene was encoded on a 7.3-kb *Eco*RI fragment from a 43-kb plasmid. To characterize this fragment, recombinant clones were constructed with partially *Eco*RI-digested *Flavobacterium* sp. plasmid DNA and *Eco*RI-digested pBR325. Clones were screened for the 7.3-kb fragment and for *opd* activity. Although no clones exhibited detectable *opd* activity, several clones were shown to contain the 7.3-kb fragment. One of these clones, which contained the recombinant plasmid pWWM44, was shown to contain the 7.3-kb *Eco*RI fragment along with a 2.3-kb *Eco*RI fragment. This clone was selected for use in the restriction mapping of the 7.3-kb *Eco*RI fragment containing the *opd* gene (Fig. 3). pWWM44 contained a 2.1-kb region (underlined area in Fig. 3) which had a restriction map identical to that for the region reported (25) to encode the *opd* gene within the analogous *Eco*RI fragment from the *P. diminuta* plasmid. This apparent homology extended at least 0.4 kb on either end of the *opd* gene. In contrast, the 3.9-kb regions to

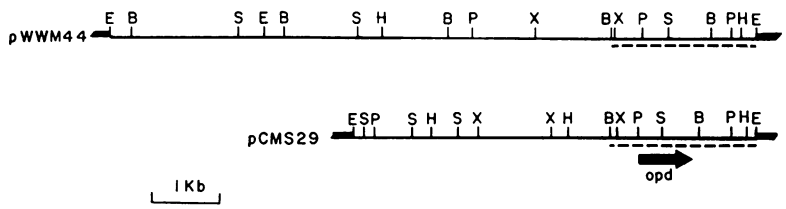

FIG. 3. Restriction endonuclease maps of cloned *Eco*RI fragments containing the *opd* genes from *P. diminuta* (from Serdar and Gibson [25], used by permission) and *Flavobacterium* sp. strain ATCC 27551. The thick lines represent the adjacent portions of vector DNA; the thin lines represent inserted plasmid DNA from *Flavobacterium* sp. (top) and *P. diminuta* (bottom). The underlined area delineates the 2.1-kb region of the two cloned fragments where the restriction maps are identical. The approximate location and orientation of the *opd* gene in *P. diminuta* is from C. S. McDaniel, J. R. Wild, and G. A. O'Donovan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, H159, p. 134, and reference 25. Restriction endonucleases: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I.

the left of the underlined area did not have identical restriction maps.

DISCUSSION

It was possible to use a cloned DNA fragment that contained the *opd* gene isolated from an American strain of *P. diminuta* to recognize the homologous DNA sequence from a Philippine *Flavobacterium* sp. We have shown that the homologous *opd* gene region in *Flavobacterium* sp. strain ATCC 27551 is encoded on a 43-kb plasmid and shares some homology with the *P. diminuta* plasmid-borne gene. Subsequent cloning and restriction mapping of an *Eco*RI fragment which contained the *Flavobacterium* sp. *opd* gene revealed that the genes had very similar restriction maps. However, the restriction maps of the cloned plasmid DNA to the left of the *opd* genes (Fig. 3) were not similar. Furthermore, the two native parathion-degrading plasmids appeared to differ in size (*Flavobacterium* sp., 43 kb; *P. diminuta*, 66 kb).

Homologous degradative plasmids from independent isolates have been documented in the literature, most notably for plasmids encoding the degradation of chlorobenzoate (9), 2,4-dichlorophenoxyacetic acid (10), and toluene (11). In general, plasmids encoding similar pathways in different organisms have been shown to be either indistinguishable in their biophysical and genetic properties or completely dissimilar. These observations have led to the suggestion (10) that the widespread occurrence of aromatic hydrocarbon-degrading plasmid-bearing strains is due to the spread of only a few ancestral plasmids through the soil microbial population. Once established, these plasmids seem to persist in different hosts relatively unchanged. Thus, in these cases, the plasmid is the unit of selection and mobility, even when the degradative genes are encoded on a transposon (e.g., TOL). Additional reports describing novel plasmids encoding the degradation of salicylate (30) and toluene (13), which show no hybridization to the previously described salicylate and toluene plasmids, support this hypothesis.

Whether the *opd*-containing plasmids will conform to the trend noted for the hydrocarbon degradation plasmids is uncertain. The two plasmids isolated to date appear to have homologous *opd* genes, as evidenced by available data on the restriction maps of their *opd*-coding regions. However, the contrasting lack of similarity between the restriction maps of plasmid regions more distant to the *opd* genes suggests that the plasmids are not completely homologous. Restriction mapping of the remainder of these plasmids and hybridization studies will help us determine the manner in which the *opd* gene is disseminated in the environment. Is the *opd* gene a mobile, selectable unit, i.e., a transposon which may be found in many unrelated plasmid or chromosomal backgrounds, or is the entire plasmid the unit of selection and mobility? We have observed that many of our environmental isolates display some degree of hydrolytic activity toward parathion. Our laboratories are in the process of probing these isolates to examine the ubiquity of this *opd* gene.

Streptomycin appeared to act as a potent plasmid curing agent in *Flavobacterium* sp. strain ATCC 27551. The reason for this phenomenon is unclear at present. It is also unclear why more conventional curing agents such as mitomycin C or novobiocin did not result in a high rate of loss of the parathion hydrolase trait in *Flavobacterium* sp. strain ATCC 27551. Unfortunately, we have not yet been successful in our efforts to obtain a derivative of *Flavobacterium* sp. strain ATCC 27551 that contains only the 43-kb plasmid bearing the *opd* gene. Isolation of such a strain will greatly facilitate the mapping and further characterization of this plasmid.

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